SIGNAL TRANSDUCTION IN CLONED MUSCARINIC RECEPTORS

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The neurotransmitter acetylcholine can recognize two receptors. These are nicotinic receptors that form ion channels and muscarinic receptors that are associated with GTP(G) proteins. Since this symposium is mainly concerned with cholinergic nicotinic receptors I will confine my introductory lecture to muscarinic receptors.

Two types of pharmacologic muscarinic receptors have been known for several years, M_1 and M_2. M_1 receptors have a high affinity for perenzepine while M_2 receptors have a low affinity for this drug. These pharmacologically define receptors have different biological responses and distribution. M_1 receptors are localized in the brain and stimulate phosphatidyl inositol metabolism while M_2 receptors are present in the

heart and inhibit adenylate cyclase. In 1987 several groups of identified investigators including our laboratory genes by expression cloning encoding five distinct muscarinic receptors. These receptors were named m1, m2, m3, m4 and m5 based on their chronological order of discovery. These receptors were found to be a member of a superfamily which span the membrane seven times and are coupled to G proteins.

Using in situ hybridization in the rat brain, the mRNA showed heterogeneous distribution of the various muscarinic subgroups. The m1, m3, and m4 muscarinic receptors ~~are~~ were found to be present in the hypocampus, striatum and dentate gyrus and are absent in the heart. The m2 receptors are present in the heart and smooth muscles but are of low abundance in the brain. The m1, and m3 receptors have been localized in the lacrimal, submandibular and parotid glands and the m2 and m3 receptors are present in the intestines, trachea and bladder. The localization of the m5 receptors have not been described.

The m1, m2, m3, m4 and m5 cloned muscarinic receptors have been

stably transfected and expressed in a mouse A9L fibroblast cells and chinese hamster ovary cells (CHO). Neither of these cell lines have an endogenous muscarinic receptor. These cell lines, transfected with five cloned muscarinic receptors, made it possible to examine the signal transduction pathways for each of the transfected muscarinic receptors.

Carbachol, a muscarinic receptor agonist inhibited cyclic AMP in transfected cells expressing m2 and m4, receptors. The carbachol induced inhibition was block by the muscarinic antagonist atropine. Cells expressing the m1, m3, m5 muscarinic receptors when stimulated with carbachol generated multiple signals. These were 1, 4, 5 inositol triphosphate ($1P_3$) and diacylglycerol generated from phospholipase C, arachidonic acid from phospholipase A_2 and phospatidic acid from phospholipase D. These muscarinic receptors also induced cyclic AMP formation and caused an elevation of cytosolic calcium. The carbachol induced second messengers were inhibited by the atropine.

The phospholipases activated by muscarinic receptors are differentially regulated. Phospholipase A_2 and D requires calcium for activation while phospholipase C does not. Phorbol esters, compounds that activate protein kinase C, augment carbachol stimulated phospholipase A_2 and arachidonic acid release. In contrast, phorbol esters pretreatment inhibited carbachol stimulated phospholipase C, ~~and~~ Carbachol generates ~~C~~ cyclic AMP indirectly via the activation of phospholipase C. The ~~ultra~~ intracellular cellular calcium generated by $1P_3$ activates calmodulin which in turn activates cylic AMP.

The muscarinic receptor (m3) stimulated increase in cytosolic calcium occurs in two phases; an initial rapid spike of ~~activation~~ followed by lower sustained increase. The initial rapid increase is caused by the release of calcium from intracellular stores by $1P_3$ as a consequence of a muscarinic receptor induced stimulation of phospholipase C. This initial rise in intracellular calcium does not require the presence of extra cellular calcium. The sustained increase requires extacellular calcium and the continued presence of carbachol. In many cell types it has been

shown that the influx of extra cellular calcium in trigger by a $1P_3$ generated intra cellular calcium. The intracellular calcium is not necessary for the influx of extracellular calcium ~~using~~ in fibroblast, A9L or CHO cells expressing cloned muscarinic receptors. When cells expressing the muscarinic m5 receptor were pretreated with phorbol esters ~~a compound~~ that inhibits both phospholipase C and the intracellular elevation of $1P_3$ also blocked the initial rise in cytosolic calcium. However the sustained slower elevation of intracellular calcium persisted as long as carbachol was present. In the absence of extracellular calcium the carbachol stimulated influx was abolished. These findings indicated that after stimulating muscarinic receptors ~~that~~ the rapid initial rise of calcium was due to release of intracellular stores of calcium by 1P3. The sustained elevation of calcium was the result of influx of extracellular calcium which was independent of intracellular calcium. The carbachol induced elevation of intracellular calcium was unaffected by inhibitors of voltage dependent calcium channels or high potassium depolarization. Thus it appears that the muscarinic receptor operated calcium channels independent of

voltage are present in CHO and A9L fibroblasts cells.

In the superfamily of seven membrane spanning receptors, the third cytosolic has been shown to represent the primary structural determinant for G-proteins. To examine the role of the third cytosolic loop in muscarinic receptors, two chimeric m2/m3 receptors were constructed in which the third cytosolic loop was exchanged between the m2 receptor and the m3 receptor in transfected A9L fibroblast cells. The wild type m2 receptor couples to inhibition of adenylate cyclase and the m3 receptors generate inositol phosphates, arachidonic acid release and cytosolic calcium elevation. Exchange of the third cytosolic loop between two receptors reverses the functional specificity of the resultant chimeric receptors. In contrast to the wild type m2 receptor which had no effect on calcium levels, the chimeric m2 receptor having a m3 cytosolic third loop construct now cause a rapid rise in calcium levels.
The chimeric m3 receptor having an m2 third loop construct failed to induce a rapid rise in calcium but stimulated the slow sustained elevation of calcium. The m3/m2 construct prevented the elevation of inositol

phosphates, arachidonic acid release and cyclic AMP generation. Thus the third cytosolic loop of the muscarinic receptors are essential for the generation of inositol phosphate, cAMP and arachidonic acid release but has no effect on the sustained calcium influx.

Phospholipase C is a member of a family of several gene products that include the isozyme phospholipase Cγ. Phospholipase Cγ is regulated by a tyrosine kinase via a receptor operated calcium channel as shown by the following experiments. Stimulation of CHO cells expressing the m5 muscarinic receptor with carbachol resulted in an increase in tyrosine phosphorylation. The concentration dependent stimulation of tyrosine phosphorylation correlated with the concentration dependent receptor stimulation of calcium influx. Inhibitors of calcium influx blocked the muscarinic stimulation of tyrosine phoshorylation. Activation of m5 muscarinic receptors also resulted in the tyrosine phosphorylation of phospholipase Cγ. Thus the phosphorylation of Cγ by muscarinic receptor activation appears to be mainly dependent on the influx of extracellular

calcium.

In synaptic vesicles ATP is copackged with the neurotransmitters, acetylcholine, norepinephrine and serotonin. Upon nerve stimulation both the neurotransmitter and ATP are coreleased. ATP, in addition to its role in intermediate metabolism, can also serve as a neurotransmitter that binds to a cell surface P_2 purinergic receptor. The interaction of ATP with P_2 purinergic receptors stimulates phospholipase A_2 to release arachidonic acid. An interaction between ATP receptors and inhibitory muscarinic receptors have been found. CHO cells have an endogenous ATP receptor ~~and~~ that can release arachidonic acid ~~when treated with ATP~~. CHO cells transfected with the inhibitory m2 or m4 receptors cannot release arachidonic acid when treated with carbachol. Stimulation of CHO cells expressing the m2 or m4 receptors with carbachol together with ATP, markedly potentiated the ATP stimulated release of arachidonic acid. Treatment with atropine blocked the carbachol induced augmentation of arachidonic acid release but had no effect on ATP evoked arachidonic acid suggesting a muscarinic receptor process. Other receptors associated

with the inhibition of adenylate cyclase including D_2 dopamine, alpha$_2$ adrenergic, and β_1 adenosine receptor also augmented the ATP generated arachidonic acid release. This potentiation phenomena by inhibitory receptors were blocked by pertussis toxin and inhibitor protein of kinase C suggesting the involvement of inhibitory G proteins and protein kinase C.